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Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT; PLUR=YES; OP=ADJ			
L8	L6 and (tail or protect$ or captur$ or immobili$).ab.	94	L8
L7	L6 and (nuclease or exonuclease or nucleolytic).ab.	35	L7
L6	L5 and (hybridi$ or probe).ab.	926	L6
L5	l4 and (nuclease or exonuclease or nucleolytic)	4824	L5
L4	L3 and (tail or reporter or protect$ or nuclease or exonuclease)	9879	L4
L3	L2 and (captur$ or immobili$)	12252	L3
L2	hybridi$	31231	L2
~~L1~~	~~hybridi$.ti or hybridi$.ab~~	~~0~~	~~L1~~

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L7: Entry 1 of 35 File: USPT Apr 9, 2002

DOCUMENT-IDENTIFIER: US 6368803 B1
TITLE: Detection of nucleic acids by target-catalyzed formation

Abstract Paragraph Left (1):
A method is disclosed for modifying an oligonucleotide, which method has application to the detection of a polynucleotide analyte. An oligonucleotide is reversibly hybridized with a polynucleotide, for example, a polynucleotide analyte, in the presence of a 5'-nuclease under isothermal conditions. The polynucleotide analyte serves as a recognition element to enable a 5'-nuclease to cleave the oligonucleotide to provide (i) a first fragment that is substantially non-hybridizable to the polynucleotide analyte and (ii) a second fragment that lies 3' of the first fragment (in the intact oligonucleotide) and is substantially hybridizable to the polynucleotide analyte. At least a 100-fold molar excess of the first fragment and/or the second fragment are obtained relative to the molar amount of the polynucleotide analyte. The presence of the first fragment and/or the second fragment is detected, the presence thereof indicating the presence of the polynucleotide analyte. The method has particular application to the detection of a polynucleotide analyte such as DNA. Kits for conducting methods in accordance with the present invention are also disclosed.

Brief Summary Paragraph Right (2):
Nucleic acid hybridization has been employed for investigating the identity and establishing the presence of nucleic acids. Hybridization is based on complementary base pairing. When complementary single stranded nucleic acids are incubated together, the complementary base sequences pair to form double stranded hybrid molecules. The ability of single stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) to form a hydrogen bonded structure with a complementary nucleic acid sequence has been employed as an analytical tool in molecular biology research. The availability of radioactive nucleoside triphosphates of high specific activity and the .sup.32 p labelling of DNA with T4 polynucleotide kinase has made it possible to identify, isolate, and characterize various nucleic acid sequences of biological interest. Nucleic acid hybridization has great potential in diagnosing disease states associated with unique nucleic acid sequences. These unique nucleic acid sequences may result from genetic or environmental change in DNA by insertions, deletions, point mutations, or by acquiring foreign DNA or RNA by means of infection by bacteria, molds, fungi, and viruses. Nucleic acid hybridization has, until now, been employed primarily in academic and industrial molecular biology laboratories. The application of nucleic acid hybridization as a diagnostic tool in clinical medicine is limited because of the frequently very low concentrations of disease related DNA or RNA present in a patient's body fluid and the unavailability of a sufficiently sensitive method of nucleic acid hybridization analysis.

Brief Summary Paragraph Right (3):
Current methods for detecting specific nucleic acid sequences generally involve immobilization of the target nucleic acid on a solid support such as nitrocellulose paper, cellulose paper, diazotized paper, or a nylon membrane. After the target nucleic acid is fixed on the support, the support is contacted with a suitably labelled probe nucleic acid for about two to forty-eight hours. After the above time period, the solid support is washed several times at a controlled temperature to remove unhybridized probe. The support is then dried and the hybridized material is detected by autoradiography or by spectrometric methods.

Brief Summary Paragraph Right (7):
Depending on which of the above amplification methods are employed, the methods generally employ from seven to twelve or more reagents. Furthermore, the above methods provide for exponential amplification of a target or a reporter

oligonucleotide. Accordingly, it is necessary to rigorously avoid contamination of assay solutions by the amplified products to avoid false positives. Some of the above methods require expensive thermal cycling instrumentation and additional reagents and sample handling steps are needed for detection of the amplified product.

Brief Summary Paragraph Right (9):
One method for detecting nucleic acids is to employ nucleic acid probes. One method utilizing such probes is described in U.S. Pat. No. 4,868,104, the disclosure of which is incorporated herein by reference. A nucleic acid probe may be, or may be capable of being, labeled with a reporter group or may be, or may be capable of becoming, bound to a support.

Brief Summary Paragraph Right (10):
Detection of signal depends upon the nature of the label or reporter group. If the label or reporter group is an enzyme, additional members of the signal producing system include enzyme substrates and so forth. The product of the enzyme reaction is preferably a luminescent product, or a fluorescent or non-fluorescent dye, any of which can be detected spectrophotometrically, or a product that can be detected by other spectrometric or electrometric means. If the label is a fluorescent molecule, the medium can be irradiated and the fluorescence determined. Where the label is a radioactive group, the medium can be counted to determine the radioactive count.

Brief Summary Paragraph Right (14):
Holland, et al., Clinical Chemistry (1992) 38:462-463, describe detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of Thermus aguaticus DNA polymerase. Longley, et al., Nucleic Acids Research (1990) 18:7317-7322, discuss characterization of the 5' to 3' exonuclease associated with Thermus aguaticus DNA polymerase. Lyamichev, et al., Science (1993) 260:778-783, disclose structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases.

Brief Summary Paragraph Right (22):
One aspect of the present invention is a method for modifying an oligonucleotide. The method comprises incubating the oligonucleotide with a polynucleotide and a 5'-nuclease wherein at least a portion of the oligonucleotide is reversibly hybridized to the polynucleotide under isothermal conditions. The oligonucleotide is cleaved to provide (i) a first fragment that is substantially non-hybridizable to the polynucleotide and includes no more than one nucleotide from the 5'-end of the portion and (ii) a second fragment that is 3' of the first fragment with reference to the intact oligonucleotide and is substantially hybridizable to the polynucleotide.

Brief Summary Paragraph Right (23):
Another aspect of the present invention is a method for detecting a polynucleotide analyte. An oligonucleotide is reversibly hybridized with a polynucleotide analyte and a 5'-nuclease under isothermal conditions. The polynucleotide analyte serves as a recognition element to enable a 5'-nuclease to cleave the oligonucleotide to provide (i) a first fragment that is substantially non-hybridizable to the polynucleotide analyte and (ii) a second fragment that lies 3' of the first fragment (in the intact oligonucleotide) and is substantially hybridizable to the polynucleotide analyte. At least a 100-fold molar excess of the first fragment and/or the second fragment are obtained relative to the molar amount of the polynucleotide analyte. The presence of the first fragment and/or the second fragment is detected, the presence thereof indicating the presence of the polynucleotide analyte.

Brief Summary Paragraph Right (24):
Another embodiment of the present invention is a method for detecting a polynucleotide analyte. A combination is provided comprising a medium suspected of containing the polynucleotide analyte, an excess, relative to the suspected concentration of the polynucleotide analyte, of a first oligonucleotide at least a portion of which is capable of reversibly hybridizing with the polynucleotide analyte under isothermal conditions, a 5'-nuclease, and a second oligonucleotide having the characteristic of hybridizing to a site on the polynucleotide analyte that is 3' of the site at which the first oligonucleotide hybridizes. The polynucleotide analyte is substantially fully hybridized to the second oligonucleotide under such isothermal conditions. The polynucleotide is reversibly hybridized under the isothermal conditions to the first oligonucleotide, which is

cleaved as a function of the presence of the polynucleotide analyte to provide, in at least a 100-fold molar excess of the polynucleotide analyte, (i) a first fragment that is substantially non-hybridizable to the polynucleotide analyte and/or (ii) a second fragment that lies 3' of the first fragment (in the intact first oligonucleotide) and is substantially hybridizable to the polynucleotide analyte. The presence of the first fragment and/or the second fragment is detected, the presence thereof indicating the presence of the polynucleotide analyte.

Brief Summary Paragraph Right (25):
Another embodiment of the present invention is a method for detecting a DNA analyte. A combination is provided comprising a medium suspected of containing the DNA analyte, a first oligonucleotide at least a portion of which is capable of reversibly hybridizing with the DNA analyte under isothermal conditions, a 5'-nuclease, and a second oligonucleotide having the characteristic of hybridizing to a site on the DNA analyte that is 3' of the site at which the first oligonucleotide hybridizes. The DNA analyte is substantially fully hybridized to the second oligonucleotide under isothermal conditions. The polynucleotide analyte is reversibly hybridized to the first oligonucleotide under isothermal conditions. The first oligonucleotide is cleaved to (i) a first fragment that is substantially non-hybridizable to the DNA analyte and (ii) a second fragment that lies 3' of the first fragment (in the intact first oligonucleotide) and is substantially hybridizable to the DNA analyte. At least a 100-fold molar excess, relative to the DNA analyte, of the first fragment and/or the second fragment is produced. The presence of the first fragment and/or the second fragment is detected, the presence thereof indicating the presence of the DNA analyte.

Brief Summary Paragraph Right (26):
Another embodiment of the present invention is a kit for detection of a polynucleotide. The kit comprises in packaged combination (a) a first oligonucleotide having the characteristic that, when reversibly hybridized under isothermal conditions to the polynucleotide, it is degraded by a 5'-nuclease to provide (i) a first fragment that is substantially non-hybridizable to the polynucleotide and (ii) a second fragment that is 3' of the first fragment (in the first oligonucleotide) and is substantially hybridizable to the polynucleotide, (b) a second oligonucleotide having the characteristic of hybridizing to a site on the polynucleotide that is separated by no more than one nucleotide from the 3'-end of the site at which the first oligonucleotide hybridizes wherein the polynucleotide is substantially fully hybridized to the second oligonucleotide under the isothermal conditions, and (c) a 5'-nuclease.

Detailed Description Paragraph Right (1):
The present invention permits catalyzed cleavage of an oligonucleotide that is modulated by a portion of a polynucleotide analyte, such as a polynucleotide, that is comprised of a target polynucleotide sequence to which a portion of the oligonucleotide hybridizes. As such, the methods of the present invention provide for very high sensitivity assays for polynucleotide analytes. The methods are simple to conduct and no temperature cycling is required. Consequently, no expensive thermal cycling instrumentation is needed. Furthermore, only a few reagents are used, thus further minimizing cost and complexity of an assay. In addition, the absence of amplified products, which are potential amplification targets, permits the use of less rigorous means to avoid contamination of assay solutions by target sequences that could produce false positives.

Detailed Description Paragraph Right (8):
Target polynucleotide sequence -- a sequence of nucleotides to be identified, which may be the polynucleotide analyte but is usually existing within a polynucleotide comprising the polynucleotide analyte. The identity of the target polynucleotide sequence is known to an extent sufficient to allow preparation of an oligonucleotide having a portion or sequence that hybridizes with the target polynucleotide sequence. In general, when one oligonucleotide is used, the oligonucleotide hybridizes with the 5'-end of the target polynucleotide sequence. When a second oligonucleotide is used, it hybridizes to a site on the target polynucleotide sequence that is 3' of the site to which the first oligonucleotide hybridizes. (It should be noted that the relationship can be considered with respect to the double stranded molecule formed when the first and second oligonucleotides are hybridized to the polynucleotide. In such context the second oligonucleotide is 5-primeward of the first oligonucleotide with respect to the "strand" comprising the first and second oligonucleotides.) The relationships described above are more clearly seen with reference to FIG. 3. The target polynucleotide sequence usually contains from

about 10 to 1,000 nucleotides, preferably 15 to 100 nucleotides, more preferably, 20 to 70 nucleotides. The target polynucleotide sequence is part of a polynucleotide that may be the entire polynucleotide analyte. The minimum number of nucleotides in the target polynucleotide sequence is selected to assure that the presence of target polynucleotide sequence in a sample is a specific indicator of the presence of polynucleotide analyte in a sample. Very roughly, the sequence length is usually greater than about 1.6 log L nucleotides where L is the number of base pairs in the genome of the biologic source of the sample. The number of nucleotides in the target sequence is usually the sum of the lengths of those portions of the oligonucleotides that hybridize with the target sequence plus the number of nucleotides lying between the portions of the target sequence that hybridize with the oligonucleotides.

Detailed Description Paragraph Right (9):
Oligonucleotide--a polynucleotide, usually a synthetic polynucleotide, usually single stranded that is constructed such that at least a portion thereof hybridizes with the target polynucleotide sequence of the polynucleotide. The oligonucleotides of this invention are usually 10 to 150 nucleotides, preferably, deoxyoligonucleotides of 15 to 100 nucleotides, more preferably, 20 to 60 nucleotides, in length.

Detailed Description Paragraph Right (10):
The first oligonucleotide, or "the" oligonucleotide when a second oligonucleotide is not employed, has a 5'-end about 0 to 100 nucleotides, preferably, 1 to 20 nucleotides in length that does not hybridize with the target polynucleotide sequence and usually has a 10 to 40 nucleotide sequence that hybridizes with the target polynucleotide sequence. In general, the degree of amplification is reduced somewhat as the length of the portion of the oligonucleotide that does not hybridize with the target polynucleotide sequence increases. The first oligonucleotide also may have a sequence at its 3'-end that does not hybridize with the target polynucleotide sequence.

Detailed Description Paragraph Right (11):
The second oligonucleotide preferably hybridizes at its 3'-end with the target polynucleotide sequence at a site on the target polynucleotide sequence 3' of the site of binding of the first oligonucleotide. The length of the portion of the second oligonucleotide that hybridizes with the target polynucleotide sequence is usually longer than the length of the portion of the first oligonucleotide that hybridizes with the target polynucleotide sequence and is usually 20 to 100 nucleotides. The melting temperature of the second oligonucleotide hybridized to the target polynucleotide sequence is preferably at least as high, more preferably, at least 5.degree. C. higher than the melting temperature of the first oligonucleotide hybridized to the target polynucleotide sequence.

Detailed Description Paragraph Right (12):
The oligonucleotides can be oligonucleotide mimics such a polynucleopeptides, phosphorothioates or phosphonates except that the first oligonucleotide usually has at least one phosphodiester bond to the nucleoside at the 5'-end of the sequence that hybridizes with the target polynucleotide sequence. When oligonucleotide mimics are used that provide very strong binding, such as polynucleopeptides, the length of the portion of the second oligonucleotide that hybridizes with the target polynucleotide sequence may be reduced to less than 20 and, preferably, greater than 10.

Detailed Description Paragraph Right (16):
Fragment--in general, in the present method the oligonucleotide (or the first oligonucleotide when a second oligonucleotide is employed) is cleaved only when at least a portion thereof is reversibly hybridized with a target polynucleotide sequence and, thus, the target polynucleotide sequence acts as a recognition element for cleavage of the oligonucleotide, thereby yielding two portions. One fragment is substantially non-hybridizable to the target polynucleotide sequence. The other fragment is substantially hybridizable to the target polynucleotide sequence and 3' of the other fragment with respect to the oligonucleotide in its uncleaved form.

Detailed Description Paragraph Right (17):
5'-Nuclease--a sequence-independent deoxyribonuclease enzyme that catalyzes the cleavage of an oligonucleotide into fragments only when at least a portion of the oligonucleotide is hybridized to the target polynucleotide sequence. The enzyme selectively cleaves the oligonucleotide near the 5'-terminus of the bound portion, within 5 nucleotides thereof, preferably within 1 to 2 nucleotides thereof and does

not cleave the unhybridized oligonucleotide or the target polynucleotide sequence. Such enzymes include both 5'-exonucleases and 5'-endonucleases but exclude ribonucleases such as RNAse H and restriction enzymes. 5'-nucleases useful in the present invention must be stable under the isothermal conditions used in the present method and are usually thermally stable nucleotide polymerases having 5'-exonuclease activity such as Taq DNA polymerase (e.g. AmpliTaq(TM) from Perkin-Elmer Corporation, Norwalk, N.J.), Thermalase Tbr(TM) DNA polymerase (from Amresco, Solon, Ohio), Ultra Therm(TM) DNA polymerase (from Bio/Can Scientific, Ontario, Canada), Replitherm(TM) DNA polymerase (from Epicentre, Madison, Wis.), Tf1(TM) DNA polymerase (from Epicentre), Panozyme(TM) DNA polymerase (from Panorama Research, Mountain View, Calif.), Tth(TM) DNA polymerase (from Epicentre), rBst(TM) DNA polymerase (from Epicentre), Heat Tuff(TM) DNA polymerase (from Clontech, Palo Alto, Calif.), and the like, derived from any source such as cells, bacteria, such as E. coli, plants, animals, virus, thermophilic bacteria, and so forth wherein the polymerase may be modified chemically or through genetic engineering to provide for thermal stability and/or increased activity.

Detailed Description Paragraph Right (18):
Isothermal conditions--a uniform or constant temperature at which the modification of the oligonucleotide in accordance with the present invention is carried out. The temperature is chosen so that the duplex formed by hybridizing the oligonucleotide-to a polynucleotide with a target polynucleotide sequence is in equilibrium with the free or unhybridized oligonucleotide and free or unhybridized target polynucleotide sequence, a condition that is otherwise referred to herein as "reversibly hybridizing" the oligonucleotide with a polynucleotide. Normally, at least 1%, preferably 20 to 80%, usually less than 95% of the polynucleotide is hybridized to the oligonucleotide under the isothermal conditions. Accordingly, under isothermal conditions there are molecules of polynucleotide that are hybridized with the oligonucleotide, or portions thereof, and are in dynamic equilibrium with molecules that are not hybridized with the oligonucleotide. Some fluctuation of the temperature may occur and still achieve the benefits of the present invention. The fluctuation generally is not necessary for carrying out the methods of the present invention and usually offer no substantial improvement. Accordingly, the term "isothermal conditions" includes the use of a fluctuating temperature, particularly random or uncontrolled fluctuations in temperature, but specifically excludes the type of fluctuation in temperature referred to as thermal cycling, which is employed in some known amplification procedures, e.g., polymerase chain reaction.

Detailed Description Paragraph Right (23):
Nucleotide polymerase--a catalyst, usually an enzyme, for forming an extension of an oligonucleotide along a polynucleotide template where the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a oligonucleotide to provide a sequence complementary with the single stranded portion of the polynucleotide to which the oligonucleotide is hybridized to form a duplex.

Detailed Description Paragraph Right (24):
Hybridization (hybridizing) and binding--in the context of nucleotide sequences these terms are used interchangeably herein. The ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific will be the binding of the two sequences. Increased stringency is achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

Detailed Description Paragraph Right (25):
Homologous or substantially identical--In general, two polynucleotide sequences that are identical or can each hybridize to the same polynucleotide sequence are homologous. The two sequences are homologous or substantially identical where the sequences each have at least 90%, preferably 100%, of the same or analogous base sequence where thymine (T) and uracil (U) are considered the same. Thus, the ribonucleotides A, U, C and G are taken as analogous to the deoxynucleotides dA, dT, dC, and dG, respectively. Homologous sequences can both be DNA or one can be DNA and the other RNA.

Detailed Description Paragraph Right (28):
Member of a specific binding pair ("sbp member")--one of two different molecules, having an area on the surface or in a cavity which specifically binds to, and is thereby defined as complementary with, a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A, DNA-DNA, DNA-RNA, and the like.

Detailed Description Paragraph Right (30):
Receptor ("antiligand")--any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, repressors, protection enzymes, protein A, complement component Clq, DNA binding proteins or ligands and the like.

Detailed Description Paragraph Right (33):
Binding of sbp members to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970). The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

Detailed Description Paragraph Right (34):
Label or reporter group or reporter molecule--a member of a signal producing system. Usually the label or reporter group or reporter molecule is conjugated to or becomes bound to, or fragmented from, an oligonucleotide or to a nucleoside triphosphate and is capable of being detected directly or, through a specific binding reaction, and can produce a detectible signal. In general, any label that is detectable can be used. The label can be isotopic or nonisotopic, usually non-isotopic, and can be a catalyst, such as an enzyme or a catalytic polynucleotide, promoter, dye, fluorescent molecule, chemiluminescer, coenzyme, enzyme substrate, radioactive group, a small organic molecule, amplifiable polynucleotide sequence, a particle such as latex or carbon particle, metal sol, crystallite, liposome, cell, etc., which may or may not be further labeled with a dye, catalyst or other detectible group, and the like. Labels include an oligonucleotide or specific polynucleotide sequence that can provide a template for amplification or ligation or act as a ligand such as for a repressor protein. The label is a member of a signal producing system and can generate a detectable signal either alone or together with other members of the signal producing system. The label can be bound directly to a nucleotide sequence or can become bound thereto by being bound to an sbp member complementary to an sbp member that is bound to a nucleotide sequence.

Detailed Description Paragraph Right (35):
Signal Producing System--The signal producing system may have one or more components, at least one component being the label or reporter group or reporter molecule. The signal producing system generates a signal that relates to the presence or amount of target polynucleotide sequence or a polynucleotide analyte in a sample. The signal producing system includes all of the reagents required to produce a measurable signal. When the label is not conjugated to a nucleotide sequence, the label is normally bound to an sbp member complementary to an sbp member that is bound to, or part of, a nucleotide sequence. Other components of the signal producing system may be included in a developer solution and can include substrates, enhancers, activators, chemiluminescent compounds, cofactors, inhibitors, scavengers, metal ions, specific binding substances required for binding of signal generating substances, and the like. Other components of the signal producing system may be coenzymes, substances that react with enzymic products, other enzymes and catalysts, and the like. The signal producing system provides a signal detectable by external means, by use of electromagnetic radiation, desirably by visual examination. The signal-producing system is described more fully in U.S. patent application Ser. No. 07/555,323, filed Jul. 19, 1990, the relevant disclosure of which is incorporated herein by reference.

Detailed Description Paragraph Right (42):
As mentioned above, the present invention has a primary application to methods for detecting a polynucleotide analyte. In one aspect of the invention an oligonucleotide is reversibly hybridized with a polynucleotide analyte in the

presence of a 5'-nuclease under isothermal conditions. In this way the polynucleotide analyte serves as a "recognition element" to enable the 5'-nuclease to specifically cleave the oligonucleotide to provide first and second fragments when the oligonucleotide is reversibly hybridized to the polynucleotide analyte. The first fragment comprises the 5'-end of the oligonucleotide (with reference to the intact or original oligonucleotide) and is substantially non-hybridizable to the polynucleotide analyte and can serve as a label. The first fragment generally includes at least a portion of that part the 5'-end of the original oligonucleotide that was not hybridized to the polynucleotide analyte when the portion of the oligonucleotide that is hybridizable with the polynucleotide analyte is reversibly hybridized thereto. Additionally, the first fragment may include nucleotides (usually, no more than 5, preferably, no more than 2, more preferably, no more than 1 of such nucleotides) that are cleaved by the 5'-nuclease from the 5'-end of that portion (or sequence) of the original oligonucleotide that was hybridized to the polynucleotide analyte. Therefore, it is in the above context that the first fragment is "substantially non-hybridizable" with the polynucleotide analyte. The second fragment comprises the sequence of nucleotides at the 3'-end of the oligonucleotide that were reversibly hybridized to the polynucleotide analyte minus those nucleotides cleaved by the 5'-nuclease when the original oligonucleotide is reversibly hybridized to the polynucleotide analyte. Accordingly, the second fragment is "substantially hybridizable" to the polynucleotide analyte having resulted from that portion of the oligonucleotide that reversibly hybridizes with the polynucleotide analyte.

Detailed Description Paragraph Right (43):
As mentioned above, the 3'-end of the oligonucleotide may include one or more nucleotides that do not hybridize with the polynucleotide analyte and may comprise a label. At least a 100-fold molar excess of the first fragment and/or the second fragment are obtained relative to the molar amount of the polynucleotide analyte. The sequence of at least one of the fragments is substantially preserved during the reaction. The presence of the first fragment and/or the second fragment is detected, the presence thereof indicating the presence of the polynucleotide analyte.

Detailed Description Paragraph Right (44):
The 5'-nuclease is generally present in an amount sufficient to cause the cleavage of the oligonucleotide, when it is reversibly hybridized to the polynucleotide analyte, to proceed at least half as rapidly as the maximum rate achievable with excess enzyme, preferably, at least 75% of the maximum rate. The concentration of the 5'-nuclease is usually determined empirically. Preferably, a concentration is used that is sufficient such that further increase in the concentration does not decrease the time for the amplification by over 5-fold, preferably 2-fold. The primary limiting factor generally is the cost of the reagent. In this respect, then, the polynucleotide analyte, or at least the target polynucleotide sequence, and the enzyme are generally present in a catalytic amount.

Detailed Description Paragraph Right (48):
The pH for the medium is usually in the range of about 4.5 to 9.5, more usually in the range of about 5.5-8.5, and preferably in the range of about 6-8. The pH and temperature are chosen so as to achieve the reversible hybridization or equilibrium state under which cleavage of an oligonucleotide occurs in accordance with the present invention. In some instances, a compromise is made in the reaction parameters in order to optimize the speed, efficiency, and specificity of these steps of the present method. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention but in individual methods one buffer may be preferred over another.

Detailed Description Paragraph Right (50):
One embodiment of the invention is depicted in FIG. 1. Oligonucleotide OL is combined with polynucleotide analyte PA having target polynucleotide sequence TPS and with a 5'-nuclease, which can be, for example, a Taq polymerase. In this embodiment OL is labeled (*) within what is designated the first fragment, produced upon cleavage of the oligonucleotide in accordance with the present invention. OL in this embodiment usually is at least 10 nucleotides in length, preferably, about 10 to 50 nucleotides in length, more preferably, 15 to 30 or more nucleotides in length. In general, the length of OL should be sufficient so that a portion hybridizes with TPS, the length of such portion approximating the length of TPS. In this embodiment the length of OL is chosen so that the cleavage of no more than 5,

preferably, no more than 1 to 3, more preferably, 1 to 2 nucleotides, therefrom results in two fragments. The first fragment, designated LN, is no more than 5 nucleotides in length, preferably, 1 to 3 nucleotides in length, more preferably, 1 to 2 nucleotides in length and the second fragment, designated DOL, is no more than 5, preferably, no more than 1 to 3, more preferably, no more than 1 to 2, nucleotides shorter than the length of OL.

Detailed Description Paragraph Right (51):
As shown in FIG. 1, OL hybridizes with TPS to give duplex I. The hybridization is carried out under isothermal conditions so that OL is reversibly hybridized with TPS. OL in duplex I is cleaved to give DOL and LN, wherein LN includes a labeled nucleotide (*) In the embodiment depicted in FIG. 1, DOL is the complement of TFS except for the nucleotides missing at the 5'-end. Since during the course of the isothermal reaction the 5'-end of PA may be cleaved at or near the 5'-end of TPS, DOL may also have 0 to 5 nucleotides at its 3'-end that overhang and cannot hybridize with the residual portion of TPS. The isothermal conditions are chosen such that equilibrium exists between duplex I and its single stranded components, namely, PA and OL. Upon cleavage of OL within duplex I, an equilibrium is also established between duplex I and its single stranded components, PA and DOL. Since OL is normally present in large excess relative to the amount of DOL formed in the reaction, there are usually many more duplexes containing OL than DOL. The reaction described above for duplex I continuously produces additional molecules of DOL.

Detailed Description Paragraph Right (52):
The reaction is allowed to continue until a sufficient number of molecules of DOL and LN are formed to permit detection of the labeled LN (LN*) and, thus, the polynucleotide analyte. In this way the enzyme-catalyzed cleavage of nucleotides from the 5'-end of OL is modulated by and, therefore, related to the presence of the polynucleotide analyte. Depending on the amount of PA present, a sufficient number of molecules for detection can be obtained where the time of reaction is from about 1 minute to 24 hours. Preferably, the reaction can be carried out in less than 5 hours. As a matter of convenience it is usually desirable to minimize the time period as long as the requisite of number of molecules of detectable fragment is achieved. In general, the time period for a given degree of cleavage can be minimized by optimizing the temperature of the reaction and using concentrations of the 5'-nuclease and the oligonucleotide that provide reaction rates near the maximum achievable with excess of these reagents. Detection of the polynucleotide analyte is accomplished indirectly by detecting the label in fragment LN*. Alternatively, DOL may be detected, for example, by using the label as a means of separating LN* and OL from the reaction mixture and then detecting the residual DOL.

Detailed Description Paragraph Right (55):
Examples of particular labels or reporter molecules and their detection can be found in U.S. patent application Ser. No. 07/555,323 filed Jul. 19, 1990, the relevant disclosure of which is incorporated herein by reference.

Detailed Description Paragraph Right (56):
Detection of the signal will depend upon the nature of the signal producing system utilized. If the label or reporter group is an enzyme, additional members of the signal producing system include enzyme substrates and so forth. The product of the enzyme reaction is preferably a luminescent product, or a fluorescent or non-fluorescent dye, any of which can be detected spectrophotometrically, or a product that can be detected by other spectrometric or electrometric means. If the label is a fluorescent molecule, the medium can be irradiated and the fluorescence determined. Where the label is a radioactive group, the medium can be counted to determine the radioactive count.

Detailed Description Paragraph Right (57):
Another embodiment of the present invention is depicted in FIG. 2. Oligonucleotide OL' has a first portion or sequence SOLL that is not hybridized to TPS' and a second portion or sequence SOL2 that is hybridized to TPS'. OL' is combined with polynucleotide analyte PA' having target polynucleotide sequence TPS' and with a 5'-endonuclease (5'-endo), which can be, for example, Taq DNA polymerase and the like. OL' and 5'-endo are generally present in concentrations as described above. In the embodiment of FIG. 2, OL' is labeled (*) within the sequence SOLL wherein SOL1 may intrinsically comprise the label or may be extrinsically labeled with a specific binding member or directly detectable labeled. The length of SOL2 is as described in the embodiment of FIG. 1. In general, the length of SOL2 should be sufficient to hybridize with TPS', usually approximating the length of TPS'. SOL1 may be any

length as long as it does not substantially interfere with the cleavage of OL' and will preferably be relatively short to avoid such interference. Usually, SOL1 is about 1 to 100 nucleotides in length, preferably, 8 to 20 nucleotides in length.

Detailed Description Paragraph Right (58):
In this embodiment the cleavage of SOL1 from SOL2 results in two fragments. Cleavage in SOL2 occurs within 5 nucleotides of the bond joining SOL1 and SOL2 in OL'. The exact location of cleavage is not critical so long as the enzyme cleaves OL' only when it is bound to TPS'. The two fragments are designated LNSOL1 and DSOL2. LNSOL1 is comprised of the 5'-end of OL' and DSOL2 is comprised of the 3'-end of OL'. The sequence of at least one of LNSOL1 and DSOL2 remains substantially intact during the cleavage reaction. As shown in FIG. 2, SOL2 of OL' hybridizes with TPS' to give duplex I'. The hybridization is carried out under isothermal conditions so that OL' is reversibly hybridized with TPS'. OL' in duplex I' is cleaved to give DSOL2 and LNSOL1, the latter of which comprises a label. In the embodiment depicted in FIG. 2, DSOL2 is the complement of TPS' except for any nucleotides missing at the 5'-end thereof as a result of the cleavage of the cleavage reaction and any nucleotides appended to the 3'-end of OL' (not shown in FIG. 2) that do not hybridize with TPS'.

Detailed Description Paragraph Right (59):
The isothermal conditions are chosen such that equilibrium exists between duplex I' and its single stranded components, i.e., PA' and OL'. Upon cleavage of OL' within duplex I' and equilibrium is also established between duplex I' and its single stranded components, PA' and DSOL2. Since OL' is normally present in large excess relative to the amount of DSOL2 formed in the reaction, there are usually many more duplexes containing OL' than DSOL2. The reaction described above for duplex I' continuously produces molecules of DSOL2 and LNSOL1. The reaction is allowed to continue until a sufficient number of molecules of DSOL2 and LNSOL1 are formed to permit detection of one or both of these fragments. In this way the enzyme-catalyzed cleavage of LNSOL1 from the 5'-end of the portion of OL' hybridized to PA' is modulated by, and therefore related to, the presence of the polynucleotide analyte. The reaction parameters and the detection of DSOL2 and/or LNSOL1 are generally as described above for the embodiment of FIG. 1.

Detailed Description Paragraph Right (61):
An embodiment using a second oligonucleotide is depicted in FIG. 3. The second oligonucleotide (OL2) hybridizes to a site TPS2 on PA" that lies 3' of the site of hybridization (TPS1) of the sequence SOL2" of the first oligonucleotide, namely, OL". In the embodiment shown OL2 fully hybridizes with TPS2. This is by way of example and not limitation. The second oligonucleotide can include nucleotides at its 5' end that are not hybridizable with the target polynucleotide sequence, but its 3'-end is preferably hybridizable. Preferably, OL2 binds to a site (TPS2) that is contiguous with the site to which SOL2" hybridizes (TPS1). However, it is within the purview of the present invention that the second oligonucleotide hybridize with PA" within 1 to 5 nucleotides, preferably, 1 nucleotide, of the site to which SOL2" hybridizes. The second oligonucleotide, OL2, is usually at least as long as, and preferably longer than, SOL2", preferably, at least 2 nucleotides longer than SOL2". In general, the second oligonucleotide is about 20-100 nucleotides in length, preferably, 30-80 nucleotides in length depending on the length of SOL2". Normally, the second oligonucleotide is chosen such that it dissociates from duplex I" at a higher temperature than that at which OL" dissociates, usually at least 3.degree. C., preferably, at least 5.degree. C. or more higher.

Detailed Description Paragraph Right (62):
The presence of OL2 in duplex I" can effect the site of cleavage of OL". In particular, when OL2 binds to PA" that is not contiguous with the SOL2" site of hybridization, the cleavage site may be shifted one or more nucleotides.

Detailed Description Paragraph Right (64):
In general and specifically in any of the embodiments of FIGS. 1 to 3 above, the 3'-end of the first oligonucleotide, for example, OL, OL' and OL", may have one or more nucleotides that do not hybridize with the target polynucleotide sequence and can serve as a label but need not do so.

Detailed Description Paragraph Right (65):
It is also within the purview of the present invention to employ a single nucleoside triphosphate in any of the above embodiments, depending on the particular 5'-endonuclease chosen for the above cleavage. The decision to use a nucleoside

triphosphate and the choice of the nucleoside triphosphate are made empirically based on its ability to accelerate the reaction in accordance with the present invention. The nucleoside triphosphate is preferably one that cannot be incorporated into the first oligonucleotide as a consequence of the binding of the oligonucleotide to the target polynucleotide sequence. In this particular embodiment the added nucleoside triphosphate is present in a concentration of 1 micromolar to 10 millimolar, preferably, 10 micromolar to 1 millimolar, more preferably, 100 micromolar to 1 millimolar. It is also within the purview of the present invention to utilize the added nucleoside triphosphate to chain extend the 3'-terminus of the second oligonucleotide to render it contiguous with the site on the target polynucleotide sequence at which the first oligonucleotide hybridizes. In this approach the second oligonucleotide serves as a polynucleotide primer for chain extension. In addition, the nucleoside triphosphate is appropriately selected to accomplish such chain extension and the 5'-nuclease is selected to also have template-dependent nucleotide polymerase activity. In any event such an approach is primarily applicable to the situation where the site of binding of this second oligonucleotide, TPS2, is separated from the site of binding of the first oligonucleotide, TPS1, by a sequence of one or more identical bases that are complementary to the added nucleotide triphosphate.

Detailed Description Paragraph Right (66):
In the embodiment of FIG. 3 the mixture containing PA", OL", the second oligonucleotide OL2 and the nucleoside triphosphate is incubated at an appropriate isothermal temperature at which OL" and PA" are in equilibrium with duplex I" wherein most of the molecules of PA" and duplex I" are hybridized to OL2. During the time when a molecule of OL" is bound to PA", the 5'-endo causes the cleavage by hydrolysis of OL"-in accordance with the present invention. When the remaining portion of cleaved oligonucleotide (DSOL2") dissociates from PA", an intact molecule of OL" becomes hybridized, whereupon the process is repeated.

Detailed Description Paragraph Right (72):
As a matter of convenience, predetermined amounts of reagents employed in the present invention can be provided in a kit in packaged combination. A kit can comprise in packaged combination (a) a first oligonucleotide having the characteristic that, when reversibly hybridized to a portion of a polynucleotide to be detected, it is degraded under isothermal conditions by a 5'-nuclease to provide (i) a first fragment that is substantially non-hybridizable to the polynucleotide and (ii) a second fragment that is 3' of the first fragment and is substantially hybridizable to the polynucleotide, (b) a second oligonucleotide having the characteristic of at least a portion thereof hybridizing to a site on the polynucleotide that is 3' of the site at which the first oligonucleotide hybridizes wherein the polynucleotide is substantially fully hybridized to such portion of the second oligonucleotide under isothermal conditions, and (c) the above 5'-nuclease. The kit can further comprise a single nucleoside triphosphate.

Detailed Description Paragraph Right (78):
The above reaction protocol was repeated using, in place of Probe 1, a labeled probe, Probe 3, (5'TCG-TGG-GAA-CAA-ACG-GCG-GAT3' (SEQ ID NO:3) prepared using a Pharmacia Gene Assembler) that had 21 nucleotides with one base at the 5'-end that was not complementary, and did not hybridize with, the target DNA. The product of this reaction was a dinucleotide, namely, 5'.sup.32 P-TC-OH (SEQ ID NO:4), that represented a 10.sup.5 -fold amplification.

Detailed Description Paragraph Right (81):
Probe 4: 5'TTA-TTT-CGT-GGG-AAC-AAA-CGG-CGG-AT3' (SEQ ID NO:5) (from Oligos Etc., Inc., Wilsonville, Oreg.). Probe 4 had 26 nucleotides with six nucleotides at its 5'-end that were not complementary, nor hybridizable with, the target DNA. Probe 4 was present in a concentration of 1 micromolar. The product of this reaction was an intact seven nucleotide fragment, namely, 5'.sup.32 P-TTATTTC-OH (SEQ ID NO:6), that represented a 1.5.times.10.sup.4 -fold amplification.

Detailed Description Paragraph Right (82):
Probe 5: 5'GAT-TAG-GAT-TAG-GAT-TAG-TCG-TGG-GAA-CAA-ACG-GCG-GAT3' (SEQ ID NO:7) was prepared using a Pharmacia Gene assembler and had 39 nucleotides with 19 nucleotides at its 5'-end that were not complementary and did not hybridize with the target DNA. The product of this reaction was an intact 20 nucleotide fragment, namely, 5'.sup.32 P-GAT-TAG-GAT-TAG-GAT-TAG-TC-OH (SEQ ID NO:8), that represented a 1.5.times.10.sup.4 -fold amplification.

Detailed Description Paragraph Right (83):
In repeating the above reactions in the absence of Probe 2, product was observed but the intensity of the spot on the polyacrylamide gel was significantly less than in the presence of Probe 2. Similar results were also observed where a 1 nucleotide space existed between the 3'-end of Probe 2 and the second probe when both probes were hybridized to the target DNA.

Detailed Description Paragraph Right (85):
The above experiments demonstrate that detectable cleavage products were generated in a target-specific manner at a single temperature using enzymes having 5'-nuclease activity and a labeled oligonucleotide. The accumulation of product was enhanced by the presence of a second oligonucleotide that was longer than the first labeled oligonucleotide and that was annealed to the target polynucleotide sequence 3' of the site of hybridization of the first labeled oligonucleotide. The reactions were carried out at temperatures very close to the melting temperature (Tm) of the labeled oligonucleotide with the target polynucleotide sequence.

Other Reference Publication (1):
Longley, et al., Characterization of the 5' to 3' exonuclease associated with Thermus aquaticus DNA polymerase, Nucleic Acids Research, 18:24, pp. 7317-7322, 1990.

Other Reference Publication (2):
Holland, et al.; Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of Thermus aquaticus, Proc. Natl. Acad. Sci. USA, 88:7276-7280, 1991.

CLAIMS:

1. A kit for detection of a polynucleotide comprising in packaged combination:

(a) a first oligonucleotide having the characteristic that, when reversibly hybridized under isothermal conditions to at least a portion of said polynucleotide, it is degraded by a 5'-nuclease to provide (i) a first fragment that is substantially non-hybridizable to said polynucleotide and (ii) a second fragment that is 3' of said first fragment in said first oligonucleotide and is substantially hybridizable to said polynucleotide, wherein said is isothermal conditions are at or near the melting temperature of a duplex comprising the oligonucleotide hybridized to the polynucleotide,

(b) a second oligonucleotide having the characteristic of hybridizing to a site on said polynucleotide that is separated by no more than one nucleotide from the 3'-end of the site at which said first oligonucleotide wherein said polynucleotide is substantially fully hybridized to said second oligonucleotide under said isothermal conditions, and

(c) a 5'-nuclease.